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Circuit-based interrogation of sleep control

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Sleep is a fundamental biological process observed widely in the animal kingdom, but the neural circuits generating sleep remain poorly understood. Understanding the brain mechanisms controlling sleep requires the identification of key neurons in the control circuits and mapping of their synaptic connections. Technical innovations over the past decade have greatly facilitated dissection of the sleep circuits. This has set the stage for understanding how a variety of environmental and physiological factors influence sleep. The ability to initiate and terminate sleep on command will also help us to elucidate its functions within and beyond the brain.

Sleep is a seemingly unproductive behavioural state that takes up a large proportion of our lives, but insufficient sleep can profoundly impair our cognitive performance during wakefulness. Long-term sleep deprivation is also linked to many other health problems, including obesity and cardiovascular diseases. At the behavioural level, sleep has been observed widely across the animal kingdom, including in worms and flies, as well as vertebrates. However, the existence of two distinct types of sleep—rapid eye movement (REM) sleep and non-REM (NREM) sleep—was previously thought to be restricted to mammals and birds and has only recently been identified in reptiles¹.

Wakefulness, NREM sleep and REM sleep can be clearly distinguished based on electroencephalogram (EEG) and electromyogram (EMG) recordings, making sleep a directly quantifiable behaviour. During wakefulness, the EEG exhibits high-frequency, low-amplitude activity ('desynchronized EEG'), and the EMG shows high muscle tone (Fig. 1a, c). In contrast, the EEG during NREM sleep is dominated by high-amplitude, low-frequency (0.5–4.5 Hz) activity ('synchronized EEG') together with sleep spindles (waxing and waning of 9–15 Hz oscillations lasting for a few seconds). REM sleep is associated with vivid dreaming; it is also called paradoxical sleep, as it is characterized by desynchronized EEG resembling that during wakefulness, but the EMG shows a complete paralysis of postural muscles². The proportions of time the animal spends in wakeful, NREM and REM states and the temporal patterns of state transitions vary widely across species³ (Fig. 1b, d). However, there are some well-conserved features. For example, animals normally enter REM sleep from NREM sleep but not directly from wakefulness.

Up until the 20th century, sleep was believed to be a passive process, caused by reduced sensory stimulation that allows our normal mental and physical activities to shut down. We now know, however, that both NREM and REM sleep are controlled by distinct neural circuits in the brain, the malfunction of which causes a variety of sleep disorders. Since the discovery of the ascending reticular activating system more than half a century ago⁴, we have learned a great deal about the neural circuits supporting wakefulness^{5,6}. In contrast, the neural mechanisms generating sleep have been far more elusive. While studies based on lesion, electrical stimulation and pharmacological manipulations (Fig. 2a, b) have implicated multiple brain regions that are important for sleep^{5,6}, which neurons are responsible for triggering and maintaining NREM or REM sleep and how they are connected to each other remain largely unknown. A main difficulty resides in the fact that the sleep-promoting neurons are often spatially intermingled with, but outnumbered by,

wake-promoting neurons, making it difficult to target them selectively for circuit analysis.

Over the past decade, several new techniques have become widely available, including optogenetics⁷, pharmacogenetics⁸, imaging with

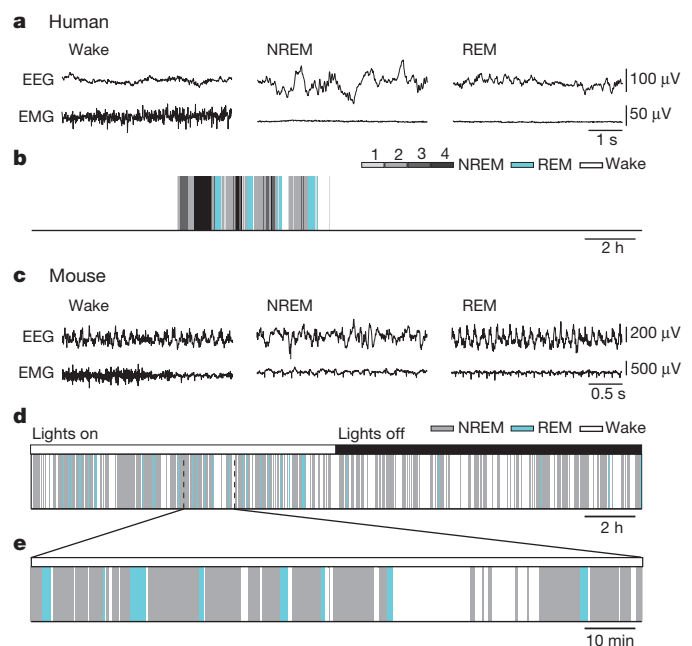


Figure 1 | Sleep in humans and mice. **a**, Examples of a human electroencephalogram (EEG) and electromyogram (EMG) recordings during wakefulness, NREM sleep (stage 3) and REM sleep. **b**, Colour-coded brain states (hypnogram) during a continuous 22-h recording from a healthy human subject. The EEG recordings (**a**) and hypnogram (**b**) are from the Sleep EDF database^{141,142}. In humans, sleep is consolidated with rare awakenings during the night. REM sleep occurs regularly every ~90 min. **c**, Example EEG and EMG recordings from a mouse during wakefulness, NREM and REM sleep. **d**, Hypnogram during a continuous 24 h recording from a mouse in a dark–light cycle. Mice sleep more during the light cycle. Compared to humans, mice exhibit fragmented sleep patterns, characterized by short sleep bouts and frequent awakenings. **e**, A 2 h segment from the hypnogram in **d** shown at an expanded scale. In mice, REM sleep occurs every 10 to 20 min.

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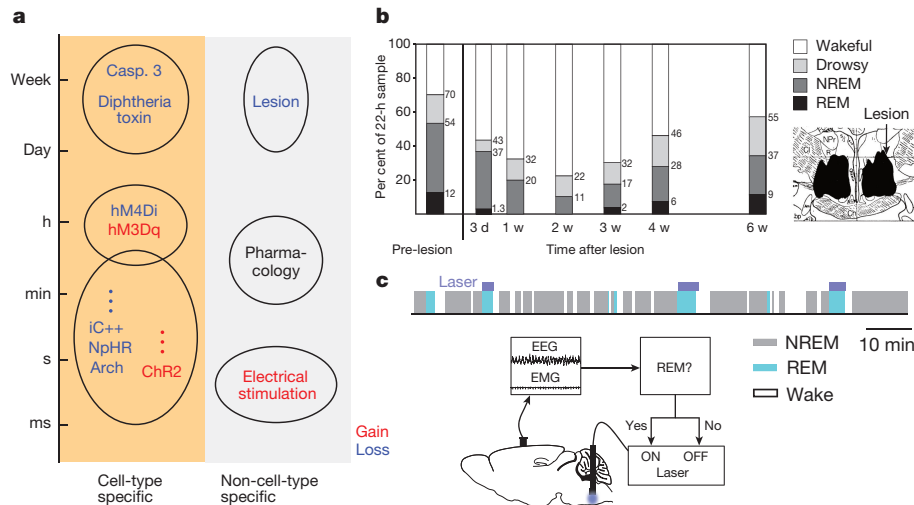


Figure 2 | Methods for neuronal manipulations. **a**, Various techniques grouped depending on whether they are cell-type-specific (orange box) or non-specific (grey box). Circle indicates timescale of each method. Text colour depicts activation versus suppression of neural activity (red and blue; gain and loss of function, respectively). Non-cell-type-specific methods include electrical stimulation of neurons or fibre tracts, pharmacological application of agonists or antagonists to specific receptors, and various methods for lesions. Using optogenetics, the light-activated cation-channel channelrhodopsin (ChR2) can be expressed in genetically defined cell types, allowing for their activation by light within milliseconds⁷. By contrast, light activation of the chloride pumps halorhodopsin (NpHR) or archaerhodopsin (Arch) causes rapid neural inhibition. Recently, a light-activated chloride channel (iC++) was developed¹⁴³. Using pharmacogenetics, neurons can be continuously

activated or inhibited for hours. The method relies on an extrinsic G protein-coupled muscarinic receptor, of which an excitatory (hM3Dq) and an inhibitory (hM4Di) version exist. The receptor is activated only by a physiologically inert, synthetic ligand⁸. Lesions of specific cell types can be achieved using genetically encoded toxins (diphtheria toxin)¹⁴⁴ or apoptotic signalling molecules (caspase 3)¹⁴⁵. **b**, Lesion of the POA and BF in cats induced long-lasting inhibition of sleep (d, days; w, weeks). Right, schematic depicting the lesioned region. Data reprinted and adapted with permission from ref. 19. **c**, Millisecond precision of neural manipulation afforded by optogenetics allowed a closed-loop stimulation protocol to test the role of GABAergic ventral medulla neurons in REM sleep maintenance. The laser was turned on after spontaneous REM onset and turned off at the end of the REM episode. Data adapted from ref. 74.

genetically encoded calcium indicators⁹ and virus-mediated circuit tracing^{10,11} (Figs 2 and 3). Combined with mouse genetics, these techniques endow us with an unprecedented capability for measuring and controlling the activity of specific cell types and dissecting their synaptic connections, greatly facilitating our investigation of the mechanisms that control sleep. In this review, we focus on the neural circuits controlling both NREM and REM sleep in the mammalian brain, with a particular emphasis on studies enabled by recently developed technologies. The function and genetics of sleep and studies in non-mammalian species are not covered here, but can be found in several recent reviews^{12–16}.

Forebrain control of sleep versus wakefulness

Multiple brain areas have been implicated in controlling the switch between wakefulness and the general state of sleep, including both REM and NREM sleep. Many of these areas are located in the forebrain, including the preoptic hypothalamus, basal forebrain and lateral hypothalamus.

Preoptic hypothalamus

The preoptic area (POA) of the anterior hypothalamus has long been known to be important for sleep generation. In the 1920s, Von Economo found that damage to the POA was associated with insomnia in human patients¹⁷. By systematically varying the location of a brain lesion in the rat, Nauta concluded that the POA is a 'sleep center'¹⁸, a notion that was supported by subsequent lesion^{19,20} and muscimol injection²¹ experiments in the cat. In the 1990s and 2000s, c-Fos immunohistochemistry following sustained sleep revealed sleep-active GABAergic neurons in the ventrolateral preoptic area (VLPO) and the median preoptic nucleus (MnPO)^{22,23}, and selective lesion of the VLPO drastically reduced NREM sleep²⁴. A recent study showed that pharmacogenetic activation of the c-Fos-labelled neurons in the POA induces sleep, further supporting their causal role in sleep regulation²⁵.

Neurons in the VLPO and MnPO are likely to promote sleep through their inhibitory projections to wake-promoting brain areas (Fig. 4).

Targets of these projections include the major wake-promoting monoaminergic centres such as the histaminergic tuberomammillary nucleus (TMN), serotonergic dorsal and median raphe nuclei (DRN and MRN) and noradrenergic locus coeruleus (LC)^{26,27}. They also project to the perifornical lateral hypothalamus²⁸, which contains the wake-promoting orexin (also known as hypocretin) neurons²⁹, the ventral periaqueductal grey matter (vPAG)³⁰ and the parabrachial nucleus²⁶, which has also been shown to be important for wakefulness and arousal³¹. Among these targets, the POA projection to the TMN in the posterior hypothalamus appears particularly strong^{26,27,32} and may powerfully inhibit TMN neurons during sleep³³. The importance of this projection is supported by the observation that inactivating the posterior hypothalamus with muscimol injection can strongly promote sleep²¹ and can reverse the insomnia induced by a POA lesion²⁰. Microdialysis measurements showed that the extracellular concentrations of GABA in the LC and DRN also increase during sleep and that GABA levels are highest during REM sleep^{34,35}, when these monoaminergic neurons are virtually silent³⁶. This shows the importance of GABAergic inputs in regulating the firing of these neurons across brain states, and it is probable that POA sleep-active neurons provide a substantial source of such GABAergic inputs. In addition to GABA, the neuropeptide galanin is also expressed in, and presumably released by, many POA sleep-active neurons^{26,27,32}, which can inhibit the TMN histaminergic neurons³⁷ and noradrenergic neurons of the LC³⁸.

What inputs activate the POA neurons during sleep and/or suppress them during wakefulness? Anatomically, histaminergic, noradrenergic and serotonergic axon fibres are observed in the POA³⁹. *In vitro* recordings showed that ~70% of VLPO neurons are inhibited by noradrenaline and acetylcholine, with a subset also inhibited by serotonin⁴⁰. Although histamine does not directly inhibit VLPO neurons, it might activate noradrenaline-excited interneurons, which could in turn inhibit the sleep neurons⁴¹. These effects of the wake-promoting neuromodulators probably suppress the activity of POA sleep neurons during wakefulness.

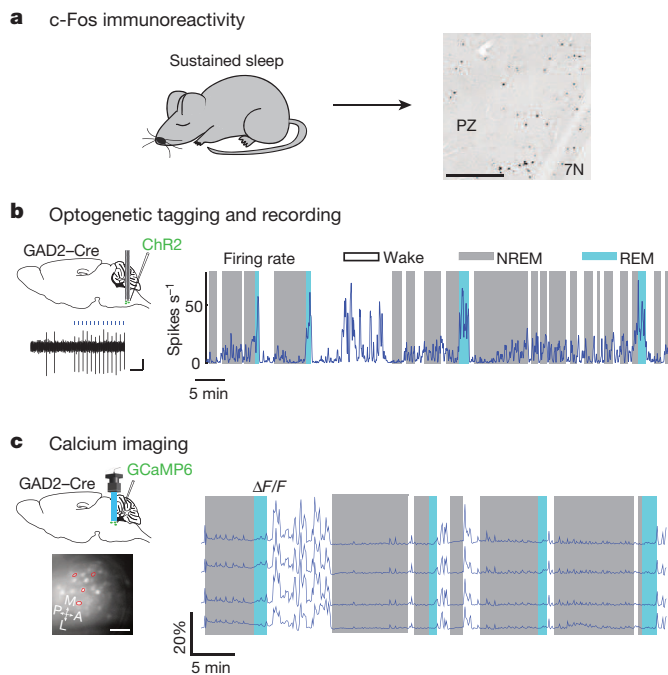


Figure 3 | Methods for measuring neural activity. **a**, *c-Fos* immunohistochemistry is widely used to detect sleep-active neurons. Following spontaneous sleep or deprivation-induced sleep rebound, brain tissue is stained for the expression of the immediate early gene *c-Fos*, used as a marker for neuronal activation. Right, brain section showing *c-Fos*-positive cells (black) in the parafacial zone (PZ; 7N, facial nucleus; scale bar, 300 μ m). Data reprinted with permission from ref. 76. **b**, Recording from genetically defined cell types using optogenetic tagging. Left, recording with optrodes (microelectrodes coupled with an optic fibre) allows the experimenter to test whether a recorded unit is reliably driven by laser stimulation and thus can be classified as the ChR2-expressing cell type. Right, example recording of a REM-active GAD2-neuron in the ventral medulla along with colour-coded brain states. Data adapted from ref. 74. **c**, Calcium imaging from identified cell types. Top left, using a microendoscope coupled to a head-mountable miniaturized camera, the calcium responses of identified cell types can be imaged in deep brain structures of freely moving mice. Bottom left, GAD2 neurons in the dorsal pons expressing the genetically encoded calcium indicator GCaMP6. Right, calcium ($\Delta F/F$) transients of five wake-active neurons (red circles in bottom left picture) along with colour-coded brain states. Data adapted from ref. 85.

Histaminergic neurons could also inhibit the sleep neurons through their co-release of GABA⁴². In addition, sleep-active POA neurons express κ and μ opioid receptors⁴³, both of which can activate potassium channels and inhibit voltage-gated calcium channels, thereby reducing the excitability of the neurons. However, while local application of a μ receptor agonist within the VLPO promotes wakefulness, a κ receptor agonist promotes NREM sleep, suggesting the existence of other sites of action besides the sleep-active neurons. Retrograde tracing combined with *in situ* hybridization suggests that neurons in the TMN release endomorphin (a μ receptor agonist), whereas those in the lateral parabrachial nucleus release dynorphin (a κ receptor agonist). Notably, dynorphin is also expressed within the POA, raising the possibility of a local source for promoting sleep.

Whereas inputs from wake-promoting neurons are generally inhibitory, VLPO neurons were recently shown to be excited by physiological concentrations of glucose and might thus mediate the sleep-promoting effect of glucose infusion into the VLPO⁴⁴. This is opposite to the wake-promoting orexin neurons, which are inhibited by high sucrose levels⁴⁵. Thus, the activity of both wake- and sleep-promoting neurons might be sensitive to the energy status of the animal, which may explain why we feel sleepy after eating a meal that is high in sugar. Moreover, the POA

contains thermosensitive neurons that are activated by either warming or cooling of the POA within a physiologically relevant temperature range⁴⁶. Notably, the majority of warm-sensitive neurons are also sleep-active⁴⁷, which may provide a mechanistic link between the control of sleep and body temperature. An important direction for future studies is to identify additional inputs to the POA sleep-promoting neurons that allow integration of temperature, energy status and other physiological variables for the optimal control of sleep.

The studies summarized above have provided important insights into how the POA contributes to sleep regulation. However, single-unit recordings and *c-Fos* staining indicate that the sleep-active neurons are not restricted to the VLPO or MnPO, and even within these regions they are spatially intermingled with wake-active neurons, many of which are also GABAergic^{48–50}. Although galanin is expressed in many VLPO sleep-active neurons^{26,27,32}, it also labels the nearby medial preoptic nucleus, which is involved in parental behaviours⁵¹. A crucial step in dissecting the POA sleep circuit is to identify molecular markers that specifically label sleep-active and sleep-promoting neurons to allow selective manipulation, recording and input and output tracing from these neurons.

Basal forebrain

While the sleep-promoting POA neurons appear to inhibit wake-promoting circuits in multiple regions of the brain, recent work in the basal forebrain (BF; adjacent to the POA) showed that sleep neurons can also suppress wake-promoting neurons locally. Lesion studies have suggested that the BF is important for both sleep and wakefulness^{19,31,52}, and it contains spatially intermingled sleep- and wake-active neurons^{49,53,54}.

There are three major cell types in the BF: cholinergic, glutamatergic and GABAergic. Juxtacellular recording and labelling in head-fixed rats followed by immunohistochemical staining showed that the cholinergic neurons are wake- and REM-active⁵⁵, and that optogenetic activation of these neurons promotes wakefulness^{56,57}. Cell-type-specific channel-rhodopsin (ChR2) tagging and optrode recording in freely moving mice showed that glutamatergic and parvalbumin (PV)-expressing GABAergic neurons are also wake- and REM-active, and that activation of these neurons promotes wakefulness. In contrast, a subpopulation of somatostatin (SOM)-expressing GABAergic neurons are NREM-active and activation of this SOM population promotes NREM sleep⁵⁷.

The intermingling of multiple cell types in the BF provides ample opportunity for local synaptic interactions, which have been analysed by ultrastructural studies⁵⁸, *in vitro* pharmacology⁵⁹ and *in vivo* microdialysis⁶⁰. Indeed, ChR2-assisted circuit mapping in BF slices revealed functional synapses for most pairs of cell types⁵⁷ (Fig. 4c). In particular, SOM GABAergic neurons provide strong inhibition to cholinergic, glutamatergic, and PV-expressing GABAergic neurons, all of which are wake-promoting. Thus, broad inhibition of multiple wake-promoting cell types, via either local synapses⁵⁷ or long-range projections^{22,26}, appears to be a common feature of sleep-promoting GABAergic neurons¹³ (Fig. 4). The local glutamatergic \rightarrow cholinergic, glutamatergic \rightarrow PV-expressing, and cholinergic \rightarrow PV-expressing neuron excitation detected in these experiments also provides useful insights into the inputs that shape the wake- and REM-active properties of these BF neurons and the local circuits that are recruited when a given cell type (for example, glutamatergic) is activated optogenetically.

Lateral hypothalamus

Similar to the POA and BF, the lateral hypothalamus also contains intermingled sleep- and wake-active neurons, with a subset of the sleep-active neurons expressing melanin-concentrating hormone (MCH)^{61,62}. A study based on juxtacellular recordings showed that MCH neurons are sleep-active, with maximal firing rates during REM sleep⁶³. Brief optogenetic activation of MCH neurons (tens of seconds per trial) increased the transitions from NREM to REM sleep⁶⁴ and prolonged the durations of REM sleep episodes⁶², indicating that MCH neuron activation enhanced both the initiation and maintenance of REM sleep. However, archaerhodopsin

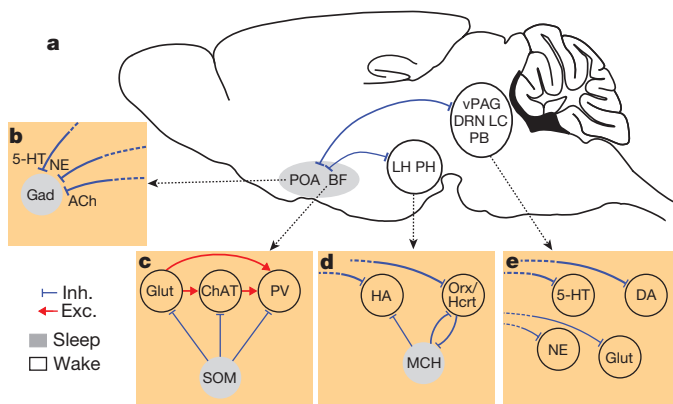


Figure 4 | Circuit diagram for forebrain sleep-promoting mechanisms. **a**, Sleep-active neurons in the POA inhibit wake-active neurons in the lateral and posterior hypothalamus (LH and PH) and in the brainstem including the dorsal raphe nucleus (DRN), locus coeruleus (LC), ventral periaqueductal grey (vPAG), and parabrachial nucleus (PB). **b**, Sleep-active GABAergic (Gad) neurons in the POA are inhibited by norepinephrine (NE), acetylcholine (ACh) and a subgroup by serotonin (5-HT). **c**, Within the basal forebrain, somatostatin (SOM) neurons inhibit neighbouring wake-promoting glutamatergic (Glut), cholinergic (ChAT), and parvalbumin (PV)-expressing GABAergic neurons. Glutamatergic neurons powerfully promote wakefulness through excitation of ChAT and PV neurons. **d**, Sleep-active neurons in the POA inhibit wake-promoting histaminergic (HA) and orexin (also known as hypocretin) (Orx/Hcrt) neurons in the LH and PH. Sleep-active melanin-concentrating hormone (MCH)-expressing neurons suppress HA and Orx/Hcrt neurons. MCH and Orx/Hcrt neurons mutually inhibit each other. **e**, Sleep-active neurons in the POA inhibit wake-promoting neurons in the brainstem including NE-, dopamine (DA)-, 5-HT- and glutamate (Glut)-expressing neurons in the LC, vPAG, DRN, and PB.

(Arch)- or halorhodopsin (NpHR)-mediated silencing of these neurons caused no pronounced change in the amount or duration of REM sleep^{62,64}, suggesting that MCH neuron activity is sufficient but perhaps not necessary. Notably, selective ablation of MCH neurons using cell-type-specific expression of diphtheria toxin A caused a decrease of NREM sleep without affecting REM sleep⁶⁴, suggesting that chronic activity of these neurons is important for NREM sleep. This notion is also supported by the finding that chronic optogenetic activation of MCH neurons (24 h) can enhance NREM as well as REM sleep⁶⁵.

The sleep-promoting effect of MCH neurons could be mediated in part by their inhibitory influence on nearby orexin neurons⁶⁶, which promote wakefulness²⁹ and indirectly inhibit MCH neurons⁶⁷ (Fig. 4d). Optogenetic activation of MCH neurons also induces inhibitory postsynaptic currents in histaminergic and other neurons in the lateral and posterior hypothalamus, a pathway that is thought to be important for the *in vivo* effect⁶². Notably, these inhibitory responses are primarily caused by GABA rather than MCH synaptic transmission, consistent with the finding that MCH neuron activation can promote REM sleep even in the absence of MCH receptors⁶².

Control of NREM versus REM sleep

After suppression of wakefulness by forebrain sleep neurons, the brain alternates between NREM and REM sleep. The duration of the so-called ultradian NREM/REM cycle varies across species; 90–120 min in humans (Fig. 1b) and 10–20 min in rodents (Fig. 1d, e). Following the discovery of REM sleep and its associated dreaming in the 1950s^{2,68}, the neural mechanisms controlling this brain state have been under active investigation.

By making surgical transections at various rostrocaudal levels of the cat brain and measuring the neural signatures of REM sleep on each side of the cut, Jouvet concluded that the brainstem is both necessary and sufficient for REM sleep generation⁶⁸. Two prominent neuromodulatory systems show opposite firing rate changes at NREM to REM transitions: monoaminergic neurons cease to fire (REM off) and cholinergic neurons

become highly active (REM on)³⁶. This led to the formulation of a model for the ultradian cycle based on the reciprocal interactions between these two neuronal populations^{36,69}. Subsequent studies, however, have pointed to more prominent roles of glutamatergic and GABAergic neurons in REM sleep generation^{70–74} (Fig. 5a). Furthermore, recent studies have identified several groups of glutamatergic and GABAergic brainstem neurons that specifically promote NREM sleep^{71,74–76} (Fig. 5b). The antagonistic interactions between these REM- and NREM-promoting neurons within the brainstem may play important roles in controlling the ultradian cycle.

REM-promoting neurons in the brainstem

Following Jouvet's landmark transection studies⁶⁸, lesion and pharmacological experiments have consistently identified the dorsolateral pons as an important region for REM sleep generation^{5,72,77,78}. This region contains diverse cell types, including cholinergic, noradrenergic, glutamatergic and GABAergic neurons, and their respective functions in REM sleep control are still under debate^{5,36,70,72}.

Cholinergic neurons in the pedunculopontine tegmentum (PPT) and laterodorsal tegmentum (LDT) are wake- and REM-active neurons⁷⁹. Early pharmacological experiments showed a powerful effect of cholinergic agonists in REM sleep generation, but application of antagonists or lesions of cholinergic neurons have yielded variable results^{5,72,80,81}. In a recent study, the role of pontine cholinergic neurons was tested using optogenetics⁸². Activation of these neurons during NREM sleep increased the frequency but not the duration of REM sleep episodes, suggesting that these neurons contribute to REM sleep initiation but perhaps not to maintenance.

Extracellular recordings in the cat suggested that there are also non-cholinergic REM-on neurons in the dorsal pons⁸³. Using c-Fos immunohistochemistry to detect REM-active cells, some investigators identified glutamatergic neurons in the sublateral dorsal nucleus (SLD)^{70,72}, while others also found GABAergic neurons in the region^{72,84}. Juxtacellular recordings in head-fixed rats confirmed the existence of REM-active GABAergic neurons⁷⁹, and cell-type-specific calcium imaging in freely moving mice revealed REM-active glutamatergic neurons⁸⁵. Pharmacological activation of the SLD induced a REM sleep-like state with EEG desynchronization and muscle atonia⁷⁷, and conditional knockout of *Vglut2* (the gene encoding vesicular glutamate transporter 2) caused fragmentation of REM sleep and reduced its amount⁸⁶, indicating an important role of SLD glutamatergic neurons in REM sleep generation.

In addition to the pons, c-Fos immunohistochemistry showed that the medulla also contains REM-active neurons⁷³. While previous studies have emphasized the function of the ventral medulla in generating the muscle atonia associated with REM sleep through its projections to the spinal cord^{87,88}, a recent study demonstrated that rostrally projecting ventral medulla neurons are critical in generating REM sleep⁷⁴. Optogenetic activation of GABAergic neurons in the ventral medulla or their axons projecting to the midbrain induced a marked increase in the probability of NREM to REM transitions, but activating glutamatergic neurons in the same region reliably induced wakefulness, attesting to the importance of cell-type-specific manipulation of neuronal activity. Using a closed-loop stimulation protocol afforded by rapid optogenetic control of neuronal activity (Fig. 2c), activation or silencing of the GABAergic neurons was found to respectively prolong or shorten the duration of each REM sleep episode, indicating that the activity of these neurons is also important for maintaining REM sleep. Furthermore, optrode recording from Chr2-tagged GABAergic neurons shows that their firing rates increase gradually over a period of tens of seconds before the onset of REM sleep and are sustained at a high level during each REM sleep episode, a temporal profile well suited for their roles in the induction and maintenance of REM sleep (Fig. 3b). Importantly, their natural firing rates during REM sleep are higher than the laser stimulation frequency necessary to induce REM sleep, indicating that their endogenous activity is sufficient for the REM-promoting effects demonstrated with optogenetic manipulations. In

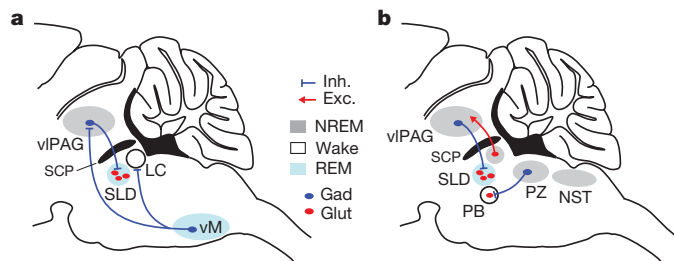


Figure 5 | Brainstem circuits controlling REM and NREM sleep.

a, Brainstem circuit promoting REM sleep, in which neuronal interactions characterized in recent studies are selectively highlighted. Glutamatergic (Glut) REM-promoting neurons in the SLD are probably inhibited by NREM-promoting GABAergic (Gad) vPAG neurons. Activation of GABAergic ventral medulla (vM) neurons that innervate the vPAG might thus disinhibit the SLD and therefore promote NREM to REM transitions. Neurons in the ventral and dorsal medulla probably inhibit noradrenergic neurons in the LC to maintain REM sleep and delay awakening. **b**, NREM-promoting circuit in the brainstem. GABAergic neurons in the vPAG, parafacial zone (PZ) and glutamatergic neurons located ventromedial to the superior cerebellar peduncle (SCP) promote NREM sleep and strongly suppress both wakefulness and REM sleep. GABAergic vPAG neurons, probably excited by the glutamatergic NREM-promoting neurons close to the SCP, inhibit the SLD and thus suppress REM sleep. Inhibition of the nearby medial parabrachial nucleus (PB) by GABAergic PZ neurons suppresses wakefulness and promotes NREM sleep. Electrical stimulation of the nucleus of the solitary tract (NTS) also increases sleep, but the underlying cell types and synaptic interactions are unknown.

addition to spike rate, such cell-type-specific recordings *in vivo* may also reveal brain-state-dependent changes in firing pattern (for example, burst versus tonic), which may strongly influence the release of neuropeptides important for regulating brain states⁸⁹.

NREM-promoting neurons in the brainstem

Early transection and pharmacological inactivation experiments pointed to a synchronizing mechanism in the medulla, which reduces the magnitude and duration of EEG desynchronization induced by stimulating the ascending reticular activating system⁹⁰. Subsequent studies showed that electrical stimulation of the nucleus of the solitary tract (NTS) synchronized the EEG and increased sleep⁹¹. The finding of NREM-active neurons in the NTS⁹² further suggests the physiological relevance of this region, although the specific cell type that mediates the synchronizing effect remains unknown.

Recent studies have identified additional cell groups in the midbrain, pons and medulla that promote NREM sleep (Fig. 5b). Pharmacogenetic activation of a population of glutamatergic neurons in the dorsolateral pons, located ventromedial to the superior cerebellar peduncle (SCP), enhanced NREM sleep⁷¹. *c-Fos* immunohistochemistry showed that many GABAergic neurons in the parafacial zone (PZ, located in the rostral medulla lateral and dorsal to the facial nucleus) are sleep-active⁷⁶. Lesion of PZ neurons or deletion of *Vgat* (the gene encoding vesicular GABA/glycine transporter) in these neurons strongly increased wakefulness, whereas pharmacogenetic activation of VGAT-expressing PZ neurons increased NREM sleep^{75,76}. This effect is thought to be mediated by inhibition of glutamatergic neurons in the medial parabrachial nucleus, which are known to be important for wakefulness and arousal³¹. Furthermore, optogenetic⁷⁴ or pharmacogenetic⁷¹ activation of GABAergic neurons in the ventrolateral periaqueductal grey (vPAG) or the adjacent deep mesencephalic reticular nucleus (DpMe) substantially increased NREM sleep, thus revealing yet another brainstem neuronal population promoting NREM sleep.

NREM/REM antagonism

Note that the hypothalamic and BF sleep neurons seem to promote NREM and/or REM sleep primarily by suppressing wakefulness (Fig. 4). In contrast, the brainstem sleep neurons discussed above also contribute

to NREM/REM antagonism. For instance, pharmacogenetic activation of PZ GABAergic neurons⁷⁵ or the glutamatergic neurons ventromedial to the SCP⁷¹ leads to a strong suppression of REM sleep whilst enhancing NREM sleep. Optogenetic⁷⁴ or pharmacogenetic⁷¹ activation of vPAG or DpMe GABAergic neurons also suppresses REM sleep in addition to wakefulness, consistent with previous lesion⁷² and pharmacological inactivation^{73,93} experiments. Conversely, activation of the ventral medulla GABAergic neurons promotes REM sleep by greatly enhancing NREM to REM transitions⁷⁴, thus effectively suppressing NREM sleep.

The circuit basis for the antagonistic relationship between NREM and REM sleep probably involves mutual inhibition between the NREM- and REM-promoting neurons. Gating of REM sleep by the vPAG and DpMe could be mediated by their GABAergic innervation of the SLD⁷², while trans-synaptic retrograde tracing with a modified rabies virus¹¹ revealed that vPAG GABAergic neurons were directly inhibited by the ventral medulla⁷⁴. Optogenetic activation of GABAergic axons of ventral medulla neurons within the vPAG was sufficient to trigger and maintain REM sleep, suggesting that the inhibition of the vPAG by the ventral medulla is an important mechanism promoting NREM to REM transitions. These inhibitory interactions between the NREM- and REM-promoting neurons might form the core of the ultradian oscillator. Notably, the duration of the ultradian cycle appears to be correlated with brain size³. In future studies it would be important to understand the temporal dynamics of the neuronal interactions that determine the duration of NREM/REM cycles, and how these dynamic properties are related to brain size.

Besides the NREM-promoting neurons, REM neurons must also interact with wake-promoting neurons. For example, GABAergic neurons in the ventral^{74,94} and dorsal⁹⁵ medulla probably inhibit the wake-active, REM-off noradrenergic neurons in the LC, which may be important for the maintenance of REM sleep by delaying awakening. Spontaneous awakening in humans is most likely to occur at the end of a REM sleep episode, and rodents typically wake up after REM sleep rather than immediately transitioning back into NREM sleep. What processes favour REM to wake over REM to NREM transitions? Notably, although some wake-promoting neurons are silent during REM sleep (for example, monoaminergic and orexinergic neurons), the majority of neurons within the dorsolateral pons are both wake- and REM-active^{79,85}. In the basal forebrain, glutamatergic, cholinergic, and PV-expressing GABAergic neurons are also REM- and wake-active, but their activation promotes wakefulness⁵⁷. Such activation of wake-promoting neurons during REM sleep might bias the transition into wake rather than NREM sleep at the end of each REM episode.

Homeostatic and circadian regulation of sleep

While the rapid transitions between wake, NREM and REM states are controlled by mutual inhibitory interactions among the neuronal groups promoting these states, sleep is also known to be regulated by homeostatic and circadian processes on much slower timescales⁹⁶. How these processes influence the sleep–wake network is only partially understood.

Sleep pressure and homeostasis

Homeostatic regulation refers to the fact that after prolonged wakefulness the animal tends to sleep for longer periods and/or at higher intensities. The increased sleep intensity is reflected by increased slow-wave activity (SWA, between 0.5 and 4.5 Hz) in the EEG, which decays gradually during the course of recovery sleep⁹⁷. SWA thus serves as an excellent marker for sleep pressure. The homeostatic regulation of sleep is under genetic control⁹⁸; a variety of genes have been shown to affect sleep homeostasis, some of which are also involved in the circadian regulation of sleep¹⁶.

More than a century ago, Ishimori and Pieron found that injection of the cerebrospinal fluid from a sleep-deprived dog into a normal one triggered sleep^{99,100}, leading to the idea that sleep pressure exerts its impact through chemical factors (somnogens) that accumulate during wakefulness. In particular, adenosine has been studied extensively as a somnogen. The extracellular concentration of adenosine increases with the time spent awake and declines during recovery sleep in both the basal

forebrain¹⁰¹ and cortex¹⁰². One source of adenosine could be the BF, with its cholinergic neurons playing a particularly important role¹⁰³. There is also strong evidence for the involvement of astrocytes in regulating the adenosine concentration^{104,105}.

Adenosine regulates neuronal activity via two major classes of adenosine receptors: the inhibitory A1 receptors that are distributed throughout the brain and the excitatory A2A receptors that are mainly localized in the striatum, nucleus accumbens and the olfactory tubercle. In A2A receptor knockout mice, caffeine (an A1 and A2A receptor antagonist) failed to promote wakefulness¹⁰⁶ and deprivation-induced NREM sleep rebound was reduced¹⁰⁷. *In vivo* application of an A2A receptor agonist increased NREM sleep and enhanced c-Fos expression in the VLPO and MnPO^{108,109}, whereas application of an antagonist in the VLPO attenuated sleep-deprivation-induced increases in the firing rates of sleep-active neurons¹¹⁰. In brain slices, VLPO neurons were shown to be activated directly or indirectly via A2A receptors^{111,112}. By contrast, A1 receptors mediate the inhibitory effect of adenosine on wake-active neurons *in vitro*, including basal forebrain cholinergic neurons¹¹³ and hypothalamic orexin neurons¹¹⁴. *In vivo* application of adenosine or A1 receptor agonists also increased NREM sleep¹¹⁵. Although A1 receptor knockout mice showed no change in the homeostatic regulation of the amount of sleep¹¹⁶, conditional deletion of A1 receptors in the forebrain and brainstem attenuated the rebound in SWA induced by sleep deprivation^{104,117}. Thus, adenosine seems to promote sleep by simultaneously activating sleep neurons through A2A receptors and suppressing wake neurons through A1 receptors.

Besides adenosine, other somnogenic factors have been identified, including prostaglandin D2, nitric oxide, growth hormone releasing hormone and cytokines (for review see ref. 118). In particular, prostaglandin D2 has been shown to be a powerful somnogen. Injection of prostaglandin D2 into the POA or the nearby subarachnoid space increases sleep and c-Fos expression in the VLPO¹¹⁹, an effect that is probably mediated via the activation of prostaglandin receptors that in turn increase adenosine levels¹²⁰.

In addition to acting on the hypothalamic and basal forebrain neurons that control global brain states, sleep homeostasis has a strong local component. Cortical regions that have been more active during the preceding wake period exhibit stronger SWA during sleep^{121,122}, which requires a local mechanism for measuring recent activity and synchronizing the cortical population. Recently, a class of cortical interneurons expressing neuronal nitric oxide synthase (nNOS) has been proposed as a link between sleep pressure and cortical SWA^{123,124}. Expression of c-Fos in nNOS neurons correlates with SWA and as these neurons have long-range intracortical projections, nNOS neurons might therefore be well suited for synchronizing the activity of neural populations¹²⁵.

While most of the studies on sleep pressure have focused on NREM sleep, REM sleep is also under strong homeostatic control¹²⁶, which is probably separate from NREM sleep homeostasis¹²⁷. The molecular and circuit mechanisms underlying REM sleep homeostasis represent an important frontier that remains largely unexplored.

Circadian rhythm of sleep

Circadian modulation of sleep depends critically on the suprachiasmatic nucleus (SCN) in the hypothalamus, the master pacemaker of the whole organism. Lesion of the SCN or its downstream target regions eliminates the daily rhythm of sleep without markedly affecting its amount^{128,129}, suggesting that the SCN is not part of the core circuit for sleep generation, but it regulates the circadian timing of sleep.

SCN neuron spiking was shown to play a key role in regulating both the molecular clock and behavioural rhythm, as optogenetic activation or suppression of SCN activity was sufficient to alter the phase and periodicity of clock gene expression and of the sleep–wake cycle¹³⁰. The firing rates of SCN neurons are high during the subjective day and low during the night, regardless of whether the animal is diurnal or nocturnal. Such circadian variation of electrical activity is controlled by both the molecular clock driven by multiple transcriptional/translational feedback

loops¹³¹ that regulates the intrinsic excitability of SCN neurons¹³² and the synaptic inputs signalling the light–dark cycle of the environment¹³³. Notably, *in vivo* multiunit recordings showed that, superimposed on the slow circadian variation, SCN neuron firing rates also change with the sleep–wake states on a timescale of seconds¹³⁴. This is probably caused by synaptic inputs from neurons involved in sleep–wake regulation, such as cholinergic and monoaminergic neurons^{135,136}. Given such ultradian firing rate modulations, it would be interesting to know whether SCN activity can exert rapid influences on brain states in the order of seconds to minutes, in addition to its well-known circadian effect in the order of hours. In addition, the SCN consists of multiple cell types including vasoactive intestinal peptide-positive and arginine vasopressin-positive cells. Whether different types of SCN neurons play distinct roles in sleep–wake regulation remains to be investigated.

The SCN projects to multiple target regions to coordinate a variety of physiological functions. Among these targets the dorsomedial hypothalamic nucleus (DMH) may play a particularly important role in sleep–wake regulation. Lesion of the DMH largely eliminated the sleep–wake circadian rhythm¹²⁸. In addition, a study using a pseudo-rabies virus for trans-synaptic retrograde tracing showed that the DMH provides an important relay from the SCN to the LC¹³⁷, which contains wake-promoting noradrenergic neurons¹³⁸. Importantly, the DMH comprises both glutamatergic and GABAergic neurons that appear to innervate both sleep- and wake-promoting circuits¹²⁸. Understanding the functional organization of this structure and how it mediates the circadian modulation of sleep again requires cell-type-specific recording, manipulation and circuit mapping.

Looking forward

The past few years have witnessed rapid progress in our understanding of the neural circuits controlling sleep, largely enabled by technical innovations that allow measurement and manipulation of neuronal activity from genetically defined cell types and tracing of their synaptic inputs and outputs. These new experimental approaches have led to the identification of additional neuronal populations in the hypothalamus, BF and brainstem that promote NREM and REM sleep, and their local and long-range connections are beginning to be delineated.

Of course, while these novel techniques have opened new avenues for investigation, it is always important to keep in mind their limitations. For example, while optogenetics provides an easily applicable method for controlling neural activity with cell-type specificity and high temporal precision (Fig. 2), the results should be interpreted with care. In sleep–wake control circuits many neurons co-release neuropeptides and other modulators together with GABA or glutamate, but the release mechanisms show differential dependence on the rate and temporal pattern (for example, burst versus tonic) of spiking⁸⁹. To assess whether the observed effects of optogenetic manipulations are physiological, it is important to measure the natural activity of the neurons across different behavioural states^{74,79,139}. Combining optogenetic manipulations with microdialysis⁶⁰ or with blockade of neurotransmitter/modulator receptors using genetic or pharmacological approaches⁶² will also provide important insights into the downstream signalling pathways mediating the behavioural effects.

One of the most exciting developments in the past few years is the identification of new sleep-promoting neurons, and the growth of this list is likely to continue. An important question is whether these sleep-promoting cell groups are organized hierarchically, in recurrent loops, or whether they work in parallel to control different aspects of sleep. NREM and REM sleep alternate in an ultradian rhythm. Although we know a great deal about how transcriptional/translational feedback loops generate the circadian rhythm and how neuronal biophysical/synaptic properties underlie network oscillations on a millisecond-to-second timescale, we know very little about how the brain generates the ultradian REM–NREM alternation on a minute-to-hour time scale. The control of REM–NREM alternations probably depends on both the synaptic interactions among spiking neurons and slower translational/transcriptional processes and

accumulation of chemical substances. Uncovering these circuit and molecular mechanisms underlying the ultradian oscillation is not only important for sleep research, but will also bridge an important gap in our understanding of brain rhythms in general.

In addition to circadian and homeostatic regulations, sleep is strongly influenced by a variety of emotional and physiological parameters, such as stress, pain, hunger and body temperature. Some of the interactions between sleep and these other processes may be mediated by common neurons shared between different control circuits. For example, while MCH neuron activity contributes to sleep regulation^{62,64,65}, the neuropeptide MCH has been implicated in feeding¹⁴⁰; and while activation of ventral medulla GABAergic neurons during NREM sleep reliably induces REM sleep, it enhances eating during wakefulness⁷⁴. Thus both hypothalamic MCH neurons and ventral medulla GABAergic neurons may help to link the regulation of sleep and feeding. The regulation of sleep by the emotional, thermal and nutritional state of the animal probably also involves synaptic inputs from these circuits to the sleep–wake control network. Furthermore, both sleep- and wake-promoting neurons are probably modulated by humoral factors such as stress hormones, cytokines and glucose. Identification of the corresponding receptors expressed in sleep and wake neurons will allow us to uncover the molecular basis for the interactions between the circuits controlling sleep and other biological functions that are essential for survival.

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